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Review

New perspectives on endothelin-1 in atherosclerosis and diabetes mellitus

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ABSTRACT

Endothelin-1 (ET-1) is a vasoconstrictor, proinflammatory and proliferative endothelial cell-derived peptide that is of significant importance in the regulation of vascular function. It is involved in the development of endothelial dysfunction including important interactions with nitric oxide. The expression and functional effects of ET-1 and its receptors are markedly altered during development of cardiovascular disease. Increased production of ET-1 and its receptors mediate many pathophysiological events contributing to the development of atherosclerosis and vascular complications in diabetes mellitus. The present review focuses on the pathophysiological role of ET-1 and the potential importance of ET receptors as a therapeutic target for treatment of these conditions.

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Introduction

Endothelial dysfunction is considered to occur early during the development of cardiovascular disease including atherosclerosis and vascular complications associated with diabetes mellitus. A key event in endothelial dysfunction is the reduction in bioavailability and biological activity of nitric oxide (NO). Reduced levels of NO contribute to increased vascular tone, inflammation, platelet aggregation and oxidative stress which all are central features of atherosclerosis and diabetic vasculopathies (Versari et al., 2009). Development of endothelial dysfunction involves several biological mediators including increased expression of endothelin (ET)-1 and altered expression of ET receptors (Böhm and Pernow, 2007). Considering the prominent biological actions mediated by ET-1 such as potent vasoconstriction, pro-inflammatory actions and mitogenic properties, overproduction of ET-1 may be of significant pathological importance in cardiovascular disease. The pathophysiological significance of ET-1

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in pulmonary arterial hypertension is well characterized (Davie et al., 2009). Recent data also indicate that ET-1 may be of importance for the pathogenesis of atherosclerosis and vascular complications associated with diabetes mellitus. Moreover, ET-1 may be involved in the metabolic features of diabetes mellitus.

ET-1 production and secretion are primarily controlled at the gene transcriptional level. ET-1 gene expression is regulated by a number of transcription factors including activator protein 1 (AP-1), hypoxia inducible factor-1, nuclear factor κB, vascular endothelial zinc finger 1 (Vezf1), GATA binding protein 2 (GATA-2) and GATA-4, nuclear factor of activated T-cells (NFAT) among others that are of relevance for atherosclerosis and diabetes. The ET-1 gene regulation has been reviewed in detail elsewhere (Stow et al., 2011). The transcription factors are in turn activated by several inducers such as angiotensin II, cytokines, glucose, insulin and hypoxia. Mature ET-1 is formed from pre-pro-ET-1 via a 39-amino acid intermediate, big ET-1 (Kedzierski et al., 2003). Big ET-1 is processed to ET-1 by a family of ET converting enzymes (ECEs) and other enzymes such as chymases, non-ECE metalloproteinases and endopeptidases (Barton et al., 2003; Kedzierski et al., 2003). Under physiological conditions, ET-1 is produced in small amounts mainly in endothelial cells, primarily acting as an autocrine and/or paracrine mediator. Under pathophysiological conditions, however, the production is stimulated in several cell types such as endothelial cells, vascular smooth muscle cells, cardiac myocytes (Ito et al., 1993) and inflammatory cells (Ehrenreich et al., 1990; Sessa et al., 1991). Increased expression of ET-1 has been demonstrated in atherosclerotic animal models (Barton et al., 1998; Lerman et al., 1993) as well as in human coronary artery disease (Lerman et al., 1991; Zeiher et al., 1994) and peripheral arterial disease (Böhm et al., 2002c). This results in enhanced vasoconstrictor tone, increased inflammatory activity and elevated oxidative stress. The effect of ET-1 is mediated via activation of its two distinct receptors, the ET_A and ET_B receptors. In the vascular wall the ET_A receptor is localized to the smooth muscle cell and mediates the major part of the vasoconstrictor effect of ET-1 under physiological conditions. The ET_B receptor is localized to the endothelial cells and mediates vasodilatation via release of NO. ET_B receptors are also located on vascular smooth muscle cells and mediate vasoconstriction. However, as pointed out below there is a substantial change in the expression and function of the ET receptors in various pathophysiological conditions resulting in an altered biological response. The present review will focus on the pathophysiological role of ET-1 in atherosclerosis and diabetes mellitus. The potential importance of ET receptors as a therapeutic target for treatment of these conditions is also discussed.

Atherosclerosis

ET-1 in the vascular wall in atherosclerosis

Several studies have demonstrated increased expression of ET-1 in experimental models of atherosclerosis as well as in human atherosclerosis (Barton et al., 1998; Lerman et al., 1993; Zeiher et al., 1994). The increased expression is observed in cells normally expressing low levels of ET-1 such as vascular smooth muscle cells and inflammatory cells. Thus, human vascular smooth muscle cells harvested from human atherosclerotic coronary arteries express higher amounts of ET-1 than cells from non-atherosclerotic arteries (Haug et al., 1996). ET-1 is associated with regions of macrophage-rich atherosclerotic plaques from patients with critical limb ischemia (Dashwood and Tsui, 2010). Targeted endothelial cell overexpression of ET-1 markedly increased atherosclerotic lesion size in apolipoprotein E gene deleted ($apoE^{-/-}$) mice fed a high fat diet and the expression of lipid metabolism genes in comparison with wild type mice (Simeone et al., 2010). This implies that increased endothelial ET-1 expression enhances lipid biosynthesis and accelerates the progression of atherosclerosis. Of clinical interest is the observation that multiple regression analysis demonstrated that the tissue ET-1 level is the main predicting factor of atherosclerosis progression in patients with chronic kidney disease (Noshad et al., 2009).

In addition, the expression of big ET-1 and ECE-1 is increased in atherosclerotic arteries (Bacon et al., 1996; Maguire and Davenport, 1998). ECE-1 was demonstrated to be localized to endothelial cells, vascular smooth muscle cells of the media, macrophages as well to the fibrous cap of atherosclerotic lesions (Böhm et al., 2002c; Ihling et al., 2001). The functional relevance of increased expression of ECE-1 in atherosclerotic arteries was investigated by determination of formation and activity of ET-1 in patients with atherosclerosis. Administration of big ET-1 resulted in more pronounced forearm vasoconstriction in patients with atherosclerosis than in age-matched controls (Böhm et al., 2002c) as well as increased local formation of ET-1. These observations suggest that the increased expression of ECE-1 translates into increased formation of ET-1 and increased vascular tone in patients with atherosclerosis.

Altered expression of ET receptors in atherosclerosis has also been described. An increased number of ET_B receptors were demonstrated in human atherosclerotic arteries (Iwasa et al., 1999). The expression was localized to inflammatory cells (macrophages, T-lymphocytes) and vascular smooth muscle cells. It was suggested that foamy macrophages and T-lymphocytes modulate a switch in expression from ET_A to ET_B receptors on vascular smooth muscle cells which may be of importance for the progression of atherosclerosis (Iwasa et al., 1999). In addition, both ET_A and ET_B receptor expression were increased in internal mammary arteries from patients with coronary artery disease (Sutherland et al., 2006). Not only receptor protein expression but also total ET-1 binding capacity for ET-1 has been described to be increased in atherosclerotic blood vessels from ($apoE^{-/-}$) mice mainly due to increased binding to the ET_A receptor (Barton et al., 1998). As illustrated in Fig. 1, available data demonstrate important alterations in the expression of ET-1 and its receptors that translate into functional involvement of ET-1 in the regulation of vascular function and progression of atherosclerosis (see below).

Hypercholesterolaemia

Hypercholesterolaemia, which is considered as the key risk factor for the development of atherosclerosis, is associated with early impairment of endothelium-dependent vasodilatation (Celermajer et al., 1994). It is also associated with elevated plasma and tissue ET-1 concentrations, which may account for the vasomotor dysfunction under this condition (Lerman et al., 1993). Accordingly, inhibition of either ET_A or both ET_A and ET_B receptors restored endothelium-dependent vasodilatation and NO production in hypercholesterolaemic pigs (Best et al., 1999) via a mechanism related to increased activity of endothelial NO synthase (eNOS). The effect on NO production induced by dual ET_A/ET_B blockade was significantly greater than that following selective ET_A antagonism (Taner et al., 2001).

There also seems to exist important interactions between oxidized low-density lipoprotein (LDL) and ET-1 of importance in atherogenesis. Oxidized and native LDL stimulates the production of ET-1 (Niemann et al., 2005). Conversely, ET-1 stimulates the uptake of oxidized LDL in endothelial cells via stimulation of the oxidized LDL receptor LOX-1 mediated by the ET_B receptor (Morawietz et al., 2001). Furthermore, ET-1 activates ET_A receptors on human vascular smooth muscle cells to yield proteoglycans with increased binding to LDL (Ballinger et al., 2009). Reduction in plasma LDL cholesterol by statins has been demonstrated to decrease the expression of pre-pro ET-1 mRNA in endothelial cells (Hernandez-Perera et al., 1998) as well as the vasoconstrictor response to ET-1 (Mraiche et al., 2005). Previous *in vitro* studies indicate that lipid-lowering treatment suppresses the expression of ET-1 in endothelial cells (Rosenson, 2001) thereby attenuating the negative effect of ET-1 on endothelial function. Furthermore, cholesterol-lowering therapy by statins further improves the beneficial effects of ET antagonism

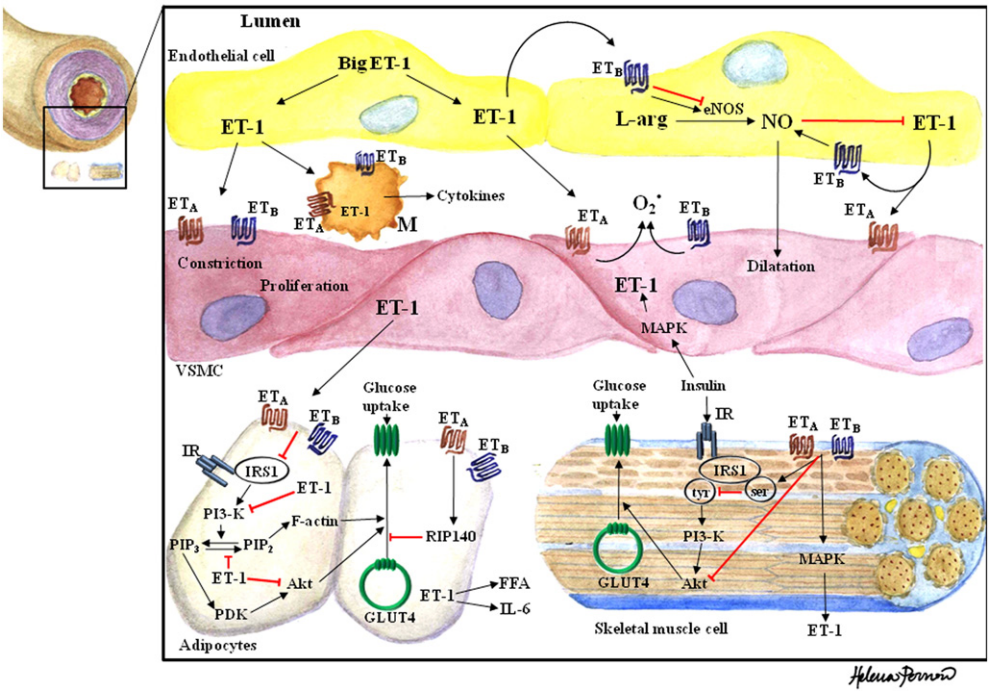

Fig. 1. Schematic figure illustrating the biological role of ET-1 in atherosclerosis and diabetes mellitus. ET-1 is under these pathophysiological situations expressed in several cell types including endothelial cells, vascular smooth muscle cells, macrophages (M) and adipocytes. ET-1 mediates vasoconstriction via activation of both ET_A and ET_B receptors on vascular smooth muscle cells and vasodilatation via stimulation of ET_B receptors and release of nitric oxide (NO) from endothelial cells. ET-1 may exert both inhibitory and stimulatory effects of endothelial NO synthase (eNOS). Additional effects of ET-1 are stimulation of vascular smooth muscle proliferation, activation of macrophages-induced cytokine production, and stimulation of superoxide production. ET-1 inhibits insulin-stimulated glucose uptake in skeletal muscle cells and adipocytes via specific receptor-dependent mechanisms at several steps down-stream of the insulin receptor (IR) and by interfering with GLUT 4 translocation. Additional effects of ET-1 in adipocytes are stimulation of lipolysis, release of free fatty acids (FFA) and pro-inflammatory cytokines like interleukin-6 (IL-6). Collectively, the effects of ET-1 result in increased vasoconstrictor tone, impaired endothelial function, oxidative stress, inflammation and insulin resistance. Additional abbreviations: IRS1, insulin receptor substrate 1; PI3-K; phosphatidylinositol 3-kinase, PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PDK, PIP_3-dependent kinase; F-actin, filamentous actin; RIP140, nuclear receptor interacting protein 140. See text for further details and references.

on NO-mediated vasodilatation in experimental hypercholesterolemia (Barton and Kiowski, 2001; Leslie et al., 2004). In a clinical study on patients with coronary artery disease and type 2 diabetes, dual ET_A/ET_B receptor blockade improved endothelium-dependent vasodilatation both before and following high dose statin therapy, suggesting that ET receptor blockade exerts beneficial effects on top of aggressive lipid lowering therapy (Settergren et al., 2008a). Collectively, these observations imply additive effect of statin therapy and ET receptor blockade on endothelial function in patients with hypercholesterolaemia and coronary artery disease.

Pro-inflammatory effects

Apart from its direct vasomotor activity, ET-1 has been implicated in inflammatory processes within the vascular wall. Specifically, ET-1 activates macrophages, resulting in the release of pro-inflammatory and chemotactic mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-8 (Browatzki et al., 2000; Hofman et al., 1998; Ruetten and Thiemermann, 1997). Cardiac overexpression of ET-1 in mice is associated with an inflammatory response involving increased activation of the pro-inflammatory transcription factor NF-κB and expression of several pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 (Yang et al., 2004). In turn, transcription factors and pro-inflammatory cytokines such as NF-κB, TNF-α, and IL-6 stimulate ET-1 production (Virdis and Schiffrin, 2003). ET-1 enhances the expression of adhesion molecules on TNF-α stimulated vascular endothelial cells (Ishizuka et al., 1999) suggesting involvement of ET_B receptors. Further, ET-1 stimulates aggregation of polymorphonuclear neutrophils (Gomez-Garre et al., 1992). Conversely, ET receptor blockade attenuates the accumulation of neutrophils and myeloperoxidase activity in the ischemic myocardium (Gonon et al., 2001). Although not a true atherosclerosis model, it has been shown that vascular inflammation and neointima formation following vascular injury by carotid artery ligation is attenuated in endothelial cell ET-1 knockout mice (Anggrahini et al., 2009).

IL-6 has been implicated in the development of atherosclerosis (Libby, 2002) and endothelial dysfunction in humans (Brull et al., 2002). As noted above, ET-1 stimulates IL-6 release *in vitro* (Browatzki et al., 2000) and *in vivo* (Böhm et al., 2007). The release of IL-6 induced by ET-1 from human vascular smooth muscle involves activation of NF-κB (Browatzki et al., 2000). Possibly, release of IL-6 may further increase oxidative stress as suggested by the *in vitro* observation that IL-6 induces production of reactive oxygen species (Wassmann et al., 2004).

Increased oxidative stress

Several reports support a role for ET-1 in the formation of reactive oxygen species (ROS). Formation of superoxide (O_2^-) will result in decreased bioactivity of NO and formation of peroxynitrite. ET-1 stimulates ROS production in human endothelial and vascular smooth muscle cell cultures (Dong et al., 2005; Duerrschmidt et al., 2000), as well as in isolated vessels (Galle et al., 2000; Loomis et al., 2005; Lopez-Sepulveda et al., 2010). Mainly ET_A receptors seem to mediate ROS production stimulated by ET-1 although ET_B receptors have been suggested to contribute to O_2^- production (Dong et al., 2005; Duerrschmidt et al., 2000). ET-1 has been shown to increase the expression of NOX2, the rate-limiting subunit of NADPH oxidase (Mohazzab et al., 1994). The stimulating effect of ET-1 on O_2^- production may also be coupled to the NADPH oxidase subunit $p22^{phox}$ (Kamata et al., 2004; Kanie and Kamata, 2002). These data are in agreement with the *in vivo* observations in transgenic mice overexpressing ET-1 (Amiri et al., 2004). These mice exhibit endothelial

dysfunction, increased NADPH oxidase activity, and increased expression of NOX2. A recent report shows that ET-1 increases expression and activity of $p47^{phox}$ in rat aortic rings via the ET_A receptor which would suggest that ET-1 is involved in the activation of NADPH oxidase (Romero et al., 2010). It was recently demonstrated that the selective ET_A antagonist avosentan significantly reduces aortic plaque formation in diabetic $apoE^{-/-}$ mice, independently of effects on blood pressure, lipid or glucose levels. The anti-atherosclerotic effect of avosentan was associated with a significant reduction in macrophage infiltration and reduced nitrotyrosine levels, reflecting a parallel decrease in oxidative stress and atherosclerosis (Watson et al., 2010). This observation supports the notion that ET-1-mediated stimulation of oxidative stress is of importance although the link between increased oxidative stress and atherosclerosis is complex as exemplified by the observation that genetic deletion of $p47^{phox}$, an essential component of NADPH oxidase, did not affect the progression of atherosclerosis in $apoE^{-/-}$ mouse model (Hsich et al., 2000). On the other hand, O_2^- generation may increase atherosclerosis by activating mitogenic signalling pathways in vascular smooth muscle cells (Vendrov et al., 2007).

The vasoconstrictor effects of ET-1 may be more pronounced in states of reduced bioavailability of the eNOS co-factor tetrahydrobiopterin (BH4) (Verma et al., 2001a). Recent data demonstrate that ET-1 mediates O_2^- production and vasoconstriction through activation of NADPH oxidase and uncoupled NOS in the rat aorta (Loomis et al., 2005). The uncoupling of NOS means that NOS generates O_2^- instead of NO in states of BH4 deficiency. These effects could be inhibited by BH4 and by dual ET receptor blockade, but not by selective ET_A receptor blockade (Loomis et al., 2005). ET-1 may also promote BH4 deficiency in a rat model of hypertension via an ET_A-mediated NADPH oxidase pathway which contributes to impaired endothelium-dependent relaxation (Zheng et al., 2003).

ET-1 has been demonstrated to be associated with increased oxidative stress and endothelial dysfunction in humans. ET-1 mediates a marked increase in O_2^- production in internal mammary arteries and saphenous veins from patients with coronary artery disease via a mechanism involving a flavin-dependent enzyme which is likely to be NADPH oxidase also in humans (Cerrato et al., 2010). ET-1 also stimulates O_2^- formation and impairs endothelium-dependent vasodilatation in human venous bypass conduits from patients with coronary artery disease and diabetes (Ergul et al., 2005). The impairment in endothelium-dependent vasodilatation *in vivo* induced by ET-1 in healthy humans can be prevented by administration of the anti-oxidant vitamin C (Böhm et al., 2007). Conversely, ET-1 is increased in human coronary artery disease by oxygen-derived radicals *ex vivo* and *in vivo* (Knappe et al., 2007), indicating a vicious circle of oxidative stress leading to increased expression of ET-1 which in turn increases oxidative stress. Taken together, these data suggest that increased oxidative stress induced by ET-1 in the vessel wall contributes to endothelial dysfunction that together with pro-inflammatory effects may be important mechanisms behind development of atherosclerosis.

Interventional studies with ET receptor antagonists

Kowala et al. (1995) demonstrated that an ET_A receptor antagonist inhibited monocyte infiltration and development of fatty streak in hypercholesterolaemic hamsters. Barton and co-workers demonstrated in $apoE^{-/-}$ mice that ET_A antagonism improves endothelium-dependent, NO-mediated relaxation and reduces atherosclerosis, which occurred concomitantly with a reduction in tissue ET-1 concentrations (Barton et al., 1998). A dual ET_A/ET_B receptor antagonist reduced foam cell formation in macrophages exposed to oxidized LDL (Babaei et al., 2000). In the same study, the ET receptor antagonist significantly inhibited the development of atherosclerosis in LDL receptor knockout mice. In addition, dual ET_A/ET_B receptor blockade attenuated angiotensin II-induced atherosclerosis development in $apoE^{-/-}$ mice without affecting blood pressure (Suen et al., 2011). Selective ET_A receptor blockade was demonstrated to reduce diabetes-associated aortic atherosclerotic burden, macrophage infiltration and nitrotyrosine staining in $apoE^{-/-}$ mice (Watson et al., 2010). Also, selective ET_A receptor blockade ameliorated vascular stenosis in a mouse model of vascular remodelling following blood flow cessation (Murakoshi et al., 2002). These data clearly suggest that ET-1 is involved in the development of atherosclerosis and that ET receptor blockade exerts anti-atherogenic effects.

The observations in experimental models have led to clinical studies using ET receptor antagonists in atherosclerosis. These studies are summarized in Table 1. Cardillo and co-workers showed that BQ123 induced a significant increase in forearm blood flow in patients with hypercholesterolemia compared to normal subjects (Cardillo et al., 2000) supporting the notion that risk factors for cardiovascular disease stimulate the ET system *in vivo*. The increase in forearm vasodilatation in response to BQ123 was attenuated by inhibition of NO generation (Verhaar et al., 1998) indicating that the effect to a major part is dependent on increased NO availability. In support of this, BQ123 improved endothelium-dependent vasodilatation in patients with atherosclerosis (Böhm et al., 2002b). In another study, dual ET_A/ET_B receptor blockade evoked greater increase in forearm blood flow in patients with atherosclerosis than in controls indicating enhanced vasoconstrictor tone mediated by ET-1 (Böhm et al., 2002a). Furthermore, the vasodilator response to dual ET_A/ET_B receptor blockade was greater than that induced by selective ET_A receptor blockade in patients with atherosclerosis, whereas the opposite was observed in control subjects. Dual ET_A/ET_B receptor antagonism improves forearm endothelium-dependent vasodilatation in patients with atherosclerosis (Böhm et al., 2005a). These observations may appear surprising considering the notion that ET_B receptors mediate release of NO and induces vasodilatation. However, this highlights the change in functional response mediated by ET_B receptor from healthy conditions under which endothelial ET_B receptors cause dilatation via NO release to the situation in atherosclerosis with increased number of vascular smooth muscle cell ET_B receptors (Iwasa et al., 1999) mediating vasoconstriction. This suggests that antagonizing both receptors may be important to achieve maximal beneficial vascular effect in patients with atherosclerosis.

Studies focusing on the coronary circulation have been performed. Selective ET_A receptor blockade increases coronary artery diameter and endothelium-dependent vasodilatation (Halcox et al., 2001; Kinlay et al., 2001) in patients with atherosclerosis. Coronary dilatation was more pronounced in severely stenotic than in angiographically normal segments (Kinlay et al., 2001). In line with this, dual ET_A/ET_B receptor blockade dilates coronary conduit and resistance vessels and improves coronary endothelial function of the epicardial coronary arteries (Böhm et al., 2008; Halcox et al., 2007). In internal mammary arteries obtained from patients undergoing coronary artery bypass graft surgery, selective ET_A blockade, selective ET_B blockade, as well as dual ET_A/ET_B receptor blockade improved endothelium-dependent vasodilatation (Verma et al., 2001b). The efficacy and safety of 6 months oral treatment with the ET_A receptor antagonist atrasentan has been evaluated in patients with coronary artery disease (Raichlin et al., 2008; Reriani et al., 2010). Atrasentan treatment reduced blood pressure and improved coronary microvascular endothelial function. Collectively, these observations suggest that ET-1 is of importance for the regulation of basal coronary vascular tone and endothelial function in patients with coronary artery disease and that ET receptor antagonism improves peripheral and coronary endothelial function in these patients. Moreover, data obtained in a clinical study (Böhm et al., 2005a), supported by observations *in vitro* (Ko et al., 2002) suggest that ET receptor antagonism exerts additional beneficial effects on endothelial function beyond that of ACE inhibitors in patients with atherosclerosis.

Table 1
Interventional studies with ET receptor antagonists in patients with atherosclerosis and diabetes mellitus.

Reference	Patients, n	Intervention	Main outcome
Cardillo et al. (2000)	Hypercholesterolemia, n = 12	BQ123 intra-brachially	Increased forearm blood flow
Kinlay et al. (2001)	Coronary artery disease, n = 8	BQ123 intra-coronary	Increased coronary artery diameter, especially in stenotic areas
Halcox et al. (2001)	Coronary artery disease, n = 44	BQ123 intra-coronary	Improved coronary endothelial function and increased blood flow
Böhm et al. (2002a)	Atherosclerosis, n = 10	BQ123 + BQ788 intra-brachially	Increased forearm blood flow more than with BQ123 alone
Böhm et al. (2002b)	Atherosclerosis, n = 10	BQ123 intra-brachially	Improved forearm endothelial function and increased blood flow
Böhm et al. (2005a)	Atherosclerosis, n = 37	BQ123 + BQ788 intra-brachially	Improved forearm endothelial function and increased blood flow
Halcox et al. (2007)	Coronary artery disease, n = 39	BQ123 + BQ788 intra-coronary	Improved coronary endothelial function and increased blood flow
Settergren et al. (2008a)	Type 2 diabetes and atherosclerosis, n = 39	BQ123 + BQ788 intra-bracially	Improved forearm endothelial + vascular smooth muscle cell function
Böhm et al. (2008)	Coronary artery disease, n = 10	BQ123 +/− BQ788 intra-coronary	Improved coronary endothelial function and increased blood flow
Raichlin et al. (2008)	Multiple cardiovascular risk factors, n = 72	Oral atrasentan for 6 months	Reduction in blood pressure
Reriani et al. (2010)	Multiple cardiovascular risk factors, n = 47	Oral atrasentan for 6 months	Improved coronary microvascular endothelial function
Adlbrecht et al. (2010)	Acute ST-elevation myocardial infarction, n = 28	BQ123 intra-venously	Reduced enzymatic infarct size
Cardillo et al. (2002)	Type 2 diabetes, n = 15	BQ123 intra-brachially	Increased forearm blood flow
Shemyakin et al. (2006)	Insulin resistance, n = 12	BQ123 + BQ788 intra-brachially	Improved forearm endothelial function
Ahlborg et al. (2007)	Obesity, insulin resistance and coronary artery disease, n = 7	BQ123 + BQ788 intra-venously during hyperinsulinaemic–euglycaemic clamp	Increased insulin sensitivity
Lteif et al. (2007)	Obesity and insulin resistance, n = 7	BQ123 into the femoral artery	Increased insulin-stimulated leg glucose uptake
Settergren et al. (2008b)	Type 2 diabetes with albuminuria, n = 10	BQ123 intra-brachially	Increased nutritive skin capillary circulation
Kalani et al. (2008)	Type 2 diabetes and critical limb ischemia, n = 6	BQ123 into the femoral artery	Increased transcutaneous oxygen tension and toe blood pressure
Shemyakin et al. (2010)	Insulin resistance, n = 11	BQ123 + BQ788 intra-brachially	Increased forearm glucose uptake and insulin-mediated glucose uptake
Mann et al. (2010)	Type 2 diabetes and overt nephropathy, n = 1392	Oral avosentan for 4 months	Reduction in albuminuria, but induction of fluid overload
Kohan et al. (2011)	Type 2 diabetes and nephropathy, n = 89	Oral atrasentan for 8 weeks	Reduction in albuminuria
Rafnsson et al. (2012)	Type 2 diabetes and microalbuminuria, n = 46	Oral bosentan for 4 weeks	Improved peripheral endothelial function

Endothelial dysfunction is an early feature during reperfusion following an episode of ischaemia (Lefer and Lefer, 1996). Ischaemia–reperfusion injury is at least partially related to impaired availability of endothelium-derived NO (Lefer and Lefer, 1996). Previous studies on experimental animal models have demonstrated that ET receptor antagonists ameliorate myocardial ischaemia–reperfusion injury by reducing infarct size and improving post-ischaemic endothelium-dependent vasodilatation (Pernow and Wang, 1997). The mechanism behind the contribution of ET-1 to ischemia–reperfusion injury may be related to increased coronary vasoconstrictor effect of ET-1 (Climent et al., 2005), increased accumulation of neutropils in the reperfused myocardium (Gonon et al., 2004), reduced expression of endothelial NO synthase (Gonon et al., 2004) and production of ROS. The effect of the dual ET_A/ET_B receptor antagonist bosentan was tested in a human model of ischaemia–reperfusion injury in the forearm. It was demonstrated that administration of bosentan inhibited the development of endothelial dysfunction following 20 min of forearm ischaemia (Böhm et al., 2005b). Adlbrecht et al. (2010) recently presented data suggesting that intravenous infusion of the ET_A receptor antagonist BQ123 reduces the enzymatic infarct size in patients with posterior wall ST elevation myocardial infarction. This clinical observation supports previous data obtained in experimental studies and clearly underlines the need for additional clinical studies evaluating the efficacy of ET receptor blockade on myocardial reperfusion injury in patients with ST elevation myocardial infarction in connection with primary percutaneous intervention.

Diabetes mellitus

Type-2 diabetes mellitus is associated with micro- and macrovascular complications leading to late diabetic complications including retinopathy, nephropathy, neuropathy, skin ulcers, coronary artery disease and peripheral artery disease. The aetiology behind vascular complications associated with diabetes is multifactorial including impairment of endothelial function with reduced bioavailability of NO, increased oxidative stress and production of advanced glycosylated end products (Jansson, 2007; Kim et al., 2006; Mather et al., 2001). Endothelial dysfunction is an early finding in insulin resistance and diabetes. It has been demonstrated that increased vasoconstrictor responsiveness due to diminished NO signaling in skeletal muscle arterioles precede the development of diabetes and hypertension in a rat model of type 2 diabetes (Lesniewski et al., 2008). Data suggesting that that ET-1 may be of importance both for vascular dysfunction and the dysregulation of glucose metabolism in insulin-resistant states and type 2 diabetes is discussed below.

Molecular interactions between ET-1 and insulin in diabetes and obesity

Insulin and its intracellular signaling pathway is a key mediator in insulin resistance and diabetes. Under physiological conditions insulin will stimulate phosphatidylinositol 3-kinase (PI3-K) pathway, involving phosphorylation of the serine/threonine kinase Akt, which results in

translocation of GLUT4 transporters and stimulation of glucose uptake (Huang et al., 2005). Phosphorylation of Akt will also directly activate eNOS leading to enhanced production of NO (Dimmeler et al., 1999; Fulton et al., 1999), to maintain normal endothelial function. These vascular actions also augment the delivery of insulin and glucose to skeletal muscle, thereby enhancing glucose uptake and utilization (Baron et al., 1995). In insulin resistance states and type-2 diabetes mellitus insulin will activate alternative signaling pathways including MAPK (Gogg et al., 2009), which besides stimulating cell growth and proliferation, also stimulates the production of ET-1 (Potenza et al., 2005). Via this mechanism the pathophysiological role of ET-1 will be enhanced in insulin resistance and type-2 diabetes (Jansson, 2007). Early clinical observations and experimental animal studies have revealed that plasma levels of ET-1 are increased in insulin resistance and type-2 diabetes (Ferri et al., 1995a, 1995b; Takahashi et al., 1990). It has also been demonstrated that plasma levels of ET-1 in type-2 diabetes correlate to impaired glucose uptake, HbA1c levels, microalbuminuria and retinopathy (Collier et al., 1992; Ferri et al., 1995b; Kawamura et al., 1992). The expression of vascular ET-1 and both ET_A and ET_B receptors is increased in various experimental models of diabetes (Kelly-Cobbs et al., 2011; Matsumoto et al., 2004). Thereby, ET-1 contributes to arterial hyper-reactivity, impaired endothelial function and remodeling in animal models of diabetes (Ergul, 2011).

Obesity is one of the major risk factors for the development of insulin resistance, diabetes and cardiovascular disease. ET-1 plasma levels are increased in obese patients with the metabolic syndrome (Ferri et al., 1997). It has been demonstrated that adipose tissue releases ET-1 and expresses both ET_A and ET_B receptors and that the expression of ET_A receptor and release of ET-1 is markedly increased in obesity (Eriksson et al., 2009; van Harmelen et al., 2008). Chronic exposure of adypocytes to ET-1 leads to the insulin receptor desensitization and decreases glucose transport through IRS-1/PI3-K/Akt signaling pathway and this effect is reversed by ET_A receptor blockade (Ishibashi et al., 2001). ET-1 also stimulates IL-6 secretion from adipocytes (Chai et al., 2009) indicating that IL-6 may mediate the effect of ET-1 on insulin resistance. In addition ET-1 stimulates leptin production in adipocytes (Xiong et al., 2001), indicating that ET-1 through leptin may affect body fat stores and feeding behavior. Furthermore, long-term incubation of adipocytes with ET-1 leads to the increased lipolysis via the ET_A receptor pathway resulting in increased free fatty acid release (Eriksson et al., 2009). The authors showed that ET-1 attenuates the anti-lipolytic effect of insulin by decreasing the expression of insulin receptor, IRS-1 and phosphodiesterase-3B through the ET_B receptor pathway (van Harmelen et al., 2008). These observations suggest that ET-1 could be a link between obesity, insulin resistance and cardiovascular disease (Fig. 1).

ET-1 and vascular dysfunction in insulin resistance and type-2 diabetes

The vasoconstrictor tone of ET-1 is enhanced in patients with type-2 diabetes via an ET_A receptor-mediated effect (Cardillo et al., 2002). ET-1 also seems to contribute to endothelial dysfunction observed in subjects with insulin resistance and patients with type-2 diabetes. Administration of ET-1 induced a marked reduction in endothelium-dependent vasodilatation in subjects with insulin resistance (Shemyakin et al., 2011). The role of endogenous ET-1 has been evaluated by infusion of ET receptor antagonists both in subjects with insulin resistance and patients with type-2 diabetes (Settergren et al., 2008a; Shemyakin et al., 2006). In both these groups dual ET receptor blockade improved endothelium-dependent vasodilatation. In patients with type 2 diabetes also endothelium-independent vasodilatation was slightly but significantly improved by dual ET receptor blockade. This observation suggests that not only endothelial function but also smooth muscle cell relaxation is impaired in insulin resistant states. In one study the effect of dual ET_A/ET_B receptor blockade and selective ET_A receptor blockade on endothelial function was compared in subjects with insulin resistance. It was found that only dual ET_A/ET_B receptor blockade, but not selective ET_A receptor blockade, improved endothelium-dependent vasodilatation (Shemyakin et al., 2006). This may imply that in this group of subjects dual ET_A/ET_B receptor blockade is more beneficial than selective ET_A receptor blockade. This observation may appear surprising, since ET_B receptors are known to stimulate NO release and blockade of this receptor would be expected to impair endothelium-dependent vasodilatation. However, it has been demonstrated that vascular smooth muscle cell ET_B receptors are increased in diabetes (Ergul, 2011; Kelly-Cobbs et al., 2011) and that ET_B receptor activation stimulates production of reactive oxygen species. Thus, there are data supporting that the ET_B receptor under pathophysiological conditions may evoke effects that resemble those mediated by the ET_A receptor. Under these conditions dual ET_A/ET_B receptor blockade may be more beneficial than selective ET_A receptor blockade and illustrates the important difference in the functional responses evoked by ET receptor stimulation and blockade between physiological and pathophysiological conditions

Another important feature of type-2 diabetes is occurrence of microvascular dysfunction. The role of ET-1 for regulation of microvascular function in patients with type-2 diabetes has been investigated by determination of cutaneous nutritive capillary function of the nail fold (Settergren et al., 2008b). Intra-arterial infusion of the ET_A receptor antagonist BQ123 induced a significant increase in baseline and peak capillary blood velocity in patients with type-2 diabetes, whereas no effect was observed in non-diabetic controls (Fig. 2). This suggests an important role for ET-1 in regulation of microvascular function in patients with type-2 diabetes and that ET_A receptor blockade markedly improves microvascular function and perfusion. This study also illustrates the important difference regarding the functional role of the ET-system in patients with developed cardiovascular disease in comparison with non-diabetic patients. A subsequent pilot study also suggests a beneficial effect of ET-receptor blockade on transcutaneous oxygen tension and toe blood pressure in patients with type-2 diabetes and a critical limb ischemia (Kalani et al., 2008).

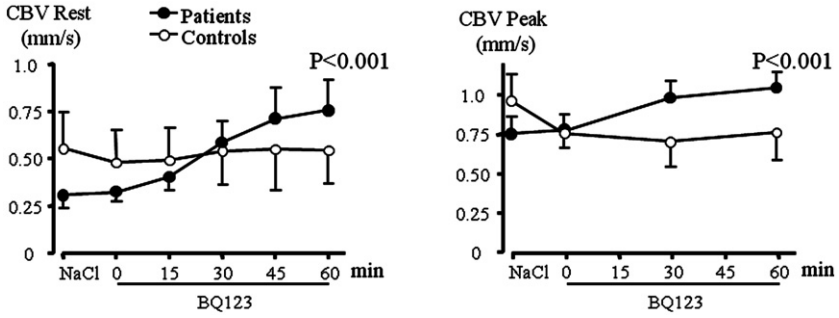

Fig. 2. Effect of ET_A receptor blockade (BQ123) on resting capillary blood cell velocity (CBV) and peak CBV following a 1 min arterial occlusion in patients with type 2 diabetes and non-diabetic controls. Data are depicted as mean and SEM. Significant differences between groups in change in resting and peak CBV following 60 min of BQ123 infusion are shown. Modified from (Settergren et al., 2008b).

Besides the aforementioned beneficial effects of ET receptor blockade following short-term intra-arterial infusions, a recent placebo-controlled double-blind study demonstrated that the dual ET_A/ET_B receptor antagonist bosentan improved peripheral endothelial function in patients with type-2 diabetes and microangiopathy (Rafnsson et al., 2012). The putative beneficial effect of long-term efficacy of ET receptor blockade on macro-and microvascular function in patients with type-2 diabetes mellitus is of considerable interest and should be further evaluated in additional randomized trials.

Since nephropathy is a common complication in type-2 diabetes and diabetic nephropathy is the leading cause of chronic kidney disease the findings regarding the role of ET-1 in diabetic nephropathy are of importance (Barton, 2010). ET receptor blockade has been demonstrated to exert beneficial effects on proteinuria in experimental studies (Watson et al., 2010). Recent clinical studies have demonstrated promising results. The non-selective ET receptor antagonist avosentan reduced albuminuria when added to standard treatment in patients with type 2 diabetes and overt nephropathy (Mann et al., 2010). However, this study was prematurely stopped due to significant fluid overload and congestive heart failure. In support of a renoprotective effect of ET receptor blockade, Kohan et al. demonstrated reduction in urine albumin/creatinine ratio in patients with type 2 diabetes and nephropathy given the ET_A selective antagonist atrasentan (Kohan et al., 2011).

Effect of ET-1 on insulin sensitivity and glucose uptake

The possibility that ET-1 may be involved in the regulation of glucose uptake either via direct effect on insulin signaling as described above or via indirect effect on peripheral blood flow is of interest. Studies on experimental models have demonstrated that ET-1 induces insulin resistance in isolated rat skeletal muscle and *in vivo* (Wilkes et al., 2003) and in adipocytes (Ishibashi et al., 2001). It has also been shown that ET-1 interferes with insulin signaling in adipocytes and skeletal muscle by reducing IRS-1 phosphorylation, translocation of GLUT4 and reduction of PI3-K (Jiang et al., 1999; Strawbridge and Elmendorf, 2005, 2006; Wilkes et al., 2003). Moreover, vascular endothelial cell-specific ET-1 knockout mice are protected from high-salt induced insulin resistance (Iwasa et al., 2010) as well as from the development of diabetes mellitus-induced cardiac fibrosis (Widyantoro et al., 2010). Conversely, insulin stimulates ET-1 production in endothelial cells via PI3K-glycogen synthase kinase-3beta (GSK3beta) signaling pathway (Yang and Li, 2008). Thus, hyperinsulinaemia observed in insulin resistance, may lead to increased ET-1 production thereby creating a vicious circle.

An initial study on humans revealed that intravenous infusion of ET-1 in healthy humans evoked a reduction in insulin sensitivity determined during a hyperinsulinaemic–euglycaemic clamp (Ottosson-Seeberger et al., 1997). Furthermore, the ET-1 precursor, big ET-1, reduced insulin sensitivity in healthy subjects via an action mediated by the ET_A receptor (Ahlborg and Lindstrom, 2002). These observations suggest that ET-1 may induce insulin resistance in humans. This was further investigated in a study on patients with insulin resistance and coronary artery disease. Intravenous infusion of dual ET_A/ET_B receptor antagonist significantly increased insulin sensitivity during a hyperinsulinaemic–euglycaemic clamp. Selective ET_A receptor blockade did not affect insulin sensitivity, suggesting a more beneficial effect of dual ET_A/ET_B receptor blockade in this patient group (Ahlborg et al., 2007). In another study (Lteif et al., 2007) described that intra-arterial infusion of an ET_A selective antagonist increased leg glucose uptake in subjects with obesity, whereas it did not affect glucose uptake in lean controls. The effect of dual ET_A/ET_B receptor blockade was not evaluated in that study. In a subsequent study performed on the forearm vascular bed in subjects with insulin resistance, it was demonstrated that intra-arterial infusion of dual ET_A/ET_B receptor antagonists increased baseline and insulin-mediated glucose uptake, suggesting improved insulin sensitivity in skeletal muscle tissue of the forearm (Fig. 3) (Shemyakin et al., 2010). Collectively, these observations indicate a role for ET-1 in the regulation of skeletal muscle glucose uptake in humans (Fig. 1). However, the interpretation of these *in vivo* studies is complicated by the fact that the interventions also induce changes in local blood flow. It is therefore difficult to conclude whether ET-1 has a direct effect on skeletal muscle glucose uptake or if the observed effects are related to changes in local blood flow and insulin delivery to skeletal muscle tissue. Data from experimental studies of isolated skeletal muscle tissue from rats support a direct effect of ET-1 on glucose uptake with an initial stimulation during short-term incubation, followed by a subsequent reduction during a prolonged exposure (Wilkes et al., 2003). Furthermore, recent data indicate a direct effect of ET-1 of glucose uptake in human skeletal muscle cells. ET-1 induced an initial increase in glucose uptake followed by reduction in glucose uptake at 24 h, which is in agreement with the data previously acquired in animal model. Western blot analyses suggest that these cells express both ET_A and ET_B receptors (Shemyakin et al., 2011).

The molecular signaling of ET-1 resulting in changes in glucose uptake has also been investigated. In human skeletal muscle cells ET-1 induced a short-term reduction in insulin-stimulated phosphorylation of Akt (Shemyakin et al., 2011). However, the inhibition of Akt phosphorylation was not observed at 24 h of ET-1 incubation suggesting that this pathway is less likely to contribute to the observed changes in glucose uptake. On the other hand ET-1 increased phosphorylation of IRS-1 at Ser636—a negative regulatory site attenuating glucose uptake. Therefore it is tempting to speculate that phosphorylation of IRS at Ser636 by ET-1 contributes to reduction in glucose uptake in skeletal muscle cells (Fig. 1). Additional pathways involved are impaired translocation of GLUT4 via phosphatidylinositol 4,5-bisphosphate (PIP2)-regulated cytoskeletal events (Strawbridge and Elmendorf, 2006) and increased cytoplasmic accumulation of RIP140 (Ho et al., 2011), which are independent of the PI3-K and Akt pathway, and G-protein couple receptor kinase-2-mediated IRS-1 Ser phosphorylation and degradation (Usui et al., 2005) . However, the signaling pathway mediating insulin resistance and reduction glucose uptake by ET-1 is far from understood and remains to be clarified in future studies.

Summary and conclusions

At present ET receptor antagonists are approved for the treatment of pulmonary arterial hypertension and for the prevention of new digital ulcers in systemic sclerosis. Accumulating evidence suggest

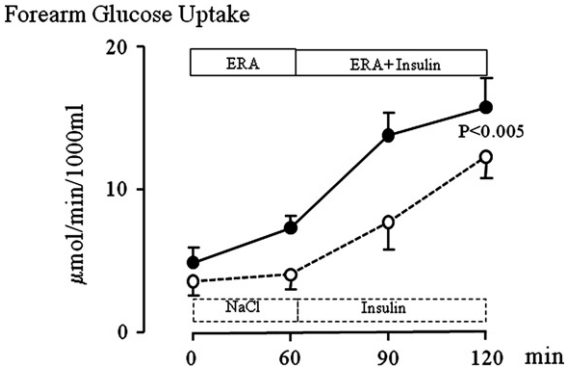

Fig. 3. Effect of dual ET_A/ET_B receptor antagonism (ERA; BQ123 and BQ788) on basal and insulin-stimulated glucose uptake in the forearm of subjects with insulin resistance. In one protocol saline was infused followed by insulin (dashed line). In another protocol saline infusion was followed by ERA alone and in combination with insulin (filled line). All infusions were given into the brachial artery. Significant difference between the entire protocols including all time points by two-way ANOVA.
Modified from Shemyakin et al. (2010).

that ET-1 is of pathophysiological importance in the development of several cardiovascular diseases including atherosclerosis, ischemic heart disease, diabetic angiopathy, diabetic nephropathy and insulin resistance. The expression of ET-1 and its receptors are markedly altered during disease progression resulting in increased biological importance of the ET-1 system (Fig. 1). Results from small randomized clinical studies support data from promising initial pilot studies. Considering the potentially important role of ET-1 in the development of vascular dysfunction reviewed in the present article—conditions with increased inflammatory activity, oxidative stress and vascular tone such as atherosclerosis, and vascular complications in diabetes—larger clinical trials using ET receptor antagonists are encouraged and needed.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2012.03.029.

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